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GENETIC CONTROL OF NON-PUNGENCY IN PEPPER (*CAPSICUM* SP.) (MINI-REVIEW)

Todorka SREBCHEVA, Bojin BOJINOV
Department of Genetics and Plant Breeding, Agricultural University, Plovdiv, Bulgaria

Abstract. The species of genus *Capsicum* are synthesizing capsaicinoids – alkaloids that give pungency in peppers. The most pungent capsaicinoids are capsaicin and dihydrocapsaicin. Capsaicin is the component of chili peppers which is irritant for mammals, including humans, and produces a sensation of burning in any tissue with which it comes into contact. Pungency as a feature has two aspects – quantitative and qualitative – affected by the way the general biosynthetic pathway of capsaicin and other capsaicinoids' synthesis is affected. Capsaicin is synthesized in the interlocular septum of plants of the *Capsicum* genus and its production is qualitatively controlled by the *Pun1* locus. It was found that the locus contains a putative acyltransferase. Several mutant alleles of *Pun1* gene were identified through classical breeding methods as responsible for the loss of pungency. Furthermore, the mutation in another locus *(Pun2)* was also found to affect the levels of capsaicin production thus providing evidence for the presence of a second gene with a major epistatic effect on capsaicin production. Here we review the current state of the knowledge accumulated so far as regards the alleles and their interactions that affect the production of this compound. The less pronounced effects of other genes in the biosynthetic pathway and some transcription factors are also discussed.

Key words: Pepper; *Capsicum*; Pungency; Genetic control.

INTRODUCTION

The *Capsicum* genus belongs to the *Solanaceae* family and includes peppers with important economic value (Giuffrida, D. et al. 2013). Following their discovery by Europeans in South and Central America at the end of the fifteenth century it took less than two hundred years for the five species of capsicum peppers to spread across the world (Halikowski-Smith, S. 2015). They are distributed broadly around the world, mainly due to their attributes of color, pungency and aroma (Bogusz Junior, S. et al. 2015; Sousa, E.T. et al. 2006).

As an economically important crop on a global scale, pepper is thoroughly researched (Das, S. et al. 2016; Ibarra-Torres, P. et al. 2015; Lahbib, K. et al. 2017; Moreira, A.F.P. et al. 2018; Tanaka, Y. et al. 2014; Tsaballa, A. et al. 2015; Zhang, X.-m. et al. 2016). One feature of particular interest of the species from *Capsicum* genus is their unique ability to synthesize capsaicin. After its biosynthesis this substance is secreted towards the outer epidermal cells and finally accumulated in structures called 'blisters' located on the placental surface (Ananthan, R. et al. 2018; Aza-González, C. et al. 2011; Lahbib, K. et al. 2017; Stewart, C. et al. 2007; Suzuki et al. 1980).

Genetic control of pungency. Pungency in peppers is due to the presence of alkaloid compound group – capsaicinoids, which are only produced in *Capsicum* genus. The most commonly occurring capsaicinoids are capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%), and homodihydrocapsaicin (1%) (Bennett and Kirby, 1968; De Aguiar et al., 2013; Lahbib, K. et al. 2017). Most pungent capsaicinoids are capsaicin and dihydrocapsaicin (both 16.0 million SHU) (Govindarajan and Sathyanarayana, 1991). Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is synthesized in placental tissues, mostly between 20 and 30 days after flowering (DAF) in the fruits of pungent pepper cultivars (Sánchez-Segura et al., 2015). John Clough Thresh first isolated capsaicin in almost pure form (Thresh, 1876) and gave it the current name. It is now well established that in mammals capsaicin activates a specific member of the transient receptor potential ligand-gated ion channel family, TRPV1 (previously known as the vanilloid receptor, VR1) (Iwai et al., 1979). The characteristic pain perceived when capsaicinoids contact tissue is a consequence of a specific interaction between the capsaicinoid molecule and the pain receptor TRPV1 (Caterina et al., 1997). As the relation between the levels of its production and the strength of pain perceived is quite straightforward it is important to be able to determine the allele states of the genes involved to distinguish pungent and sweet peppers in breeding programs.

Capsaicin ((E)-N-[(4-Hydroxy-3-methoxyphenyl) methyl]-8-methylnon-6-enamide) is the most abundant in quantity, though not more spicier than other capsaicinoids such as dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin based on the Scoville scale (Scoville, 1912). Capsaicin was first

isolated from paprika and cayenne in the late 19th century (Thresh, 1876). The exact nature of capsaicin was established in 1923 (Nelson and Dawson, 1923).

The general biosynthetic pathway of capsaicin and other capsaicinoids was elucidated in late 1960s (Bennett and Kirby, 1968); (Leete and Louden, 1968). Nowadays it is known that two pathways are involved in capsaicin biosynthesis: (1) the phenylpropanoid pathway derived from phenylalanine leading to vanillylamine; and (2) the branched chain fatty acid pathway derived from valine leading to 8-methyl-6-nonenoyl-CoA (Aza-González, C. et al. 2011; Kim, S. et al. 2014; Mazourek, M. et al. 2009). The condensation reaction of vanillylamine with 8-methyl-6-nonenoyl-CoA is thought to be catalyzed by a coenzyme A-dependent acyltransferase (Fig. 1).

Early genetic studies identified a single dominant gene, C, now known as *Pun1*, that in the homozygous recessive condition results in absence of pungency regardless of genotype at other loci throughout the genome that affect pungency level or other aspects of this trait (Deshpande, R.B.1935; Stewart, C. et al. 2005). Although the genes involved in capsaicinoid synthesis have been extensively studied, the gene responsible for the acylation of vanillylamine remained unknown until Kim et al. (Kim, M.W. et al. 2001) reported the SB2-66 clone as a possible candidate.

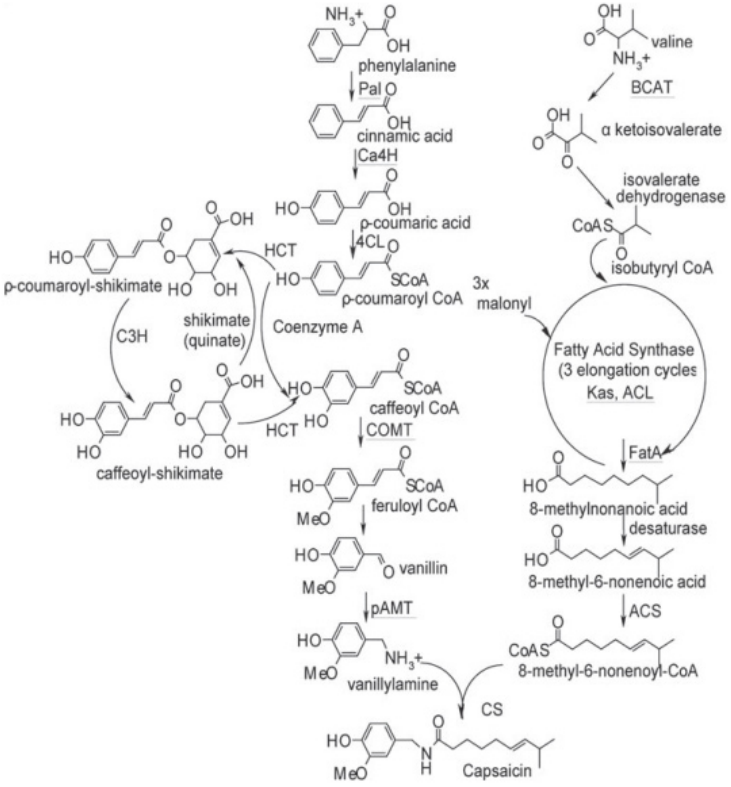

Figure 1. *Model of the capsaicinoid biosynthetic pathway*

Enzymes are shown adjacent to the reactions they catalyze. For those enzymes underlined, the gene encoding them has been cloned in *Capsicum*. Kas is the only enzyme functionally characterized in *Capsicum*; all other enzyme attributions are based on nucleotide similarity and differential gene expression. **Pal**, phenylalanine ammonia lyase; **Ca4H**, cinnamic acid 4-hydroxylase; **4CL**, 4-coumarate CoA ligase; **HCT**, hydroxycinnamoyl transferase; **C3H**, coumaroyl shikimate/quinate 3-hydroxylase; **COMT**, caffeic acid O-methyltransferase; **pAMT**, aminotransferase; **BCAT**, branched-chain amino acid transferase; **Kas**, 3-keto-acyl ACP synthase; **ACL**, acyl carrier protein; **Fat**, acyl-ACP thioesterase; **ACS**, acyl-CoA synthetase; **CS**, capsaicin synthase (Stewart, C. et al. 2007).

The C locus was first tagged by the molecular marker CD25 on linkage group 2 (Tanksley, S.D. et al. 1988). Later, more closely linked markers were developed by several research groups (Blum, E. et al. 2002; Blum, E. et al. 2003; Sugita, T. et al. 2005). To identify candidate genes for *Pun1*, differential expression patterns of transcripts in placental tissues were compared between pungent and nonpungent

peppers (Aluru, M.R. et al. 2003; Kim, M.W. et al. 2001). Among the candidate genes, the SB2-66 clone, which is specifically expressed only in the pungent placenta and encodes a putative acyltransferase (AT3), co-segregated with the *Pun1* locus (Stewart, C. et al. 2005). This study finally demonstrated that SB2-66 was the putative acyltransferase involved in capsaicinoid production. It is encoded by AT3 gene, namely Pungent gene 1 (*Pun1*), earlier known as capsaicin synthesis (CS), on chromosome 2 (Ben-Chaim, A. et al. 2001; Blum, E. et al., 2002; Lefebvre, V. et al. 1995). The expression profile of *Pun1* correlates with pepper pungency and the deletion or down-regulation of the *Pun1* gene results in a decreased accumulation of capsaicinoids.

Pun1 has been reported to control capsaicinoid synthesis in nonpungent pepper (Han, K. et al. 2013; Kobata, K. et al. 2013). The locus has a qualitative effect on capsaicinoid biosynthesis in cultivated varieties belonging to the species *C. annuum*, *C. chinense* and *C. frutescens*, which are thought to form a closely related species complex (Carvalho, S.I.C. et al., 2014). Mutations in the *Pun1* gene result in a loss of pungency.

Several mutant alleles are identified as responsible for the loss of pungency. Four loss-of-function alleles of *Pun1* have been reported to date: *pun1*^{1} in *C. annuum*, *pun1*^{2} in *C. chinense*, *pun1*^{3} in *C. frutescens* and *pun1*^{4} in the Japanese non-pungent cultivar 'Nara Murasaki' (*C. annuum*).

The allele *pun1*^{1} is widely distributed in sweet *C. annuum* cultivars. The recessive *pun1*^{1} was found to have a 2.5-kb deletion spanning from the putative promoter region to most of the first exon region (Stewart, C. et al. 2005) in which case the protein is not transcribed or translated (Fig. 2). It is thought that the abundantly distributed *pun1*^{1} allele utilized in different breeding programs involving non-pungent peppers has occurred approximately 3 centuries ago (Boswell, V.R. 1937; Lee, C.-J. et al. 2005).

The second type of *Pun1* allele, *pun1*^{2} in *C. chinense*, has a 4-bp deletion in the first exon region that creates an early stop codon. In this allele, there is transcription, but no protein product is recovered (Stewart, C. et al. 2007). Genetic analysis revealed that *pun1*^{2} co-segregated exactly with the absence of blisters, non-pungency, and a reduced transcript accumulation of several genes involved in capsaicinoid biosynthesis.

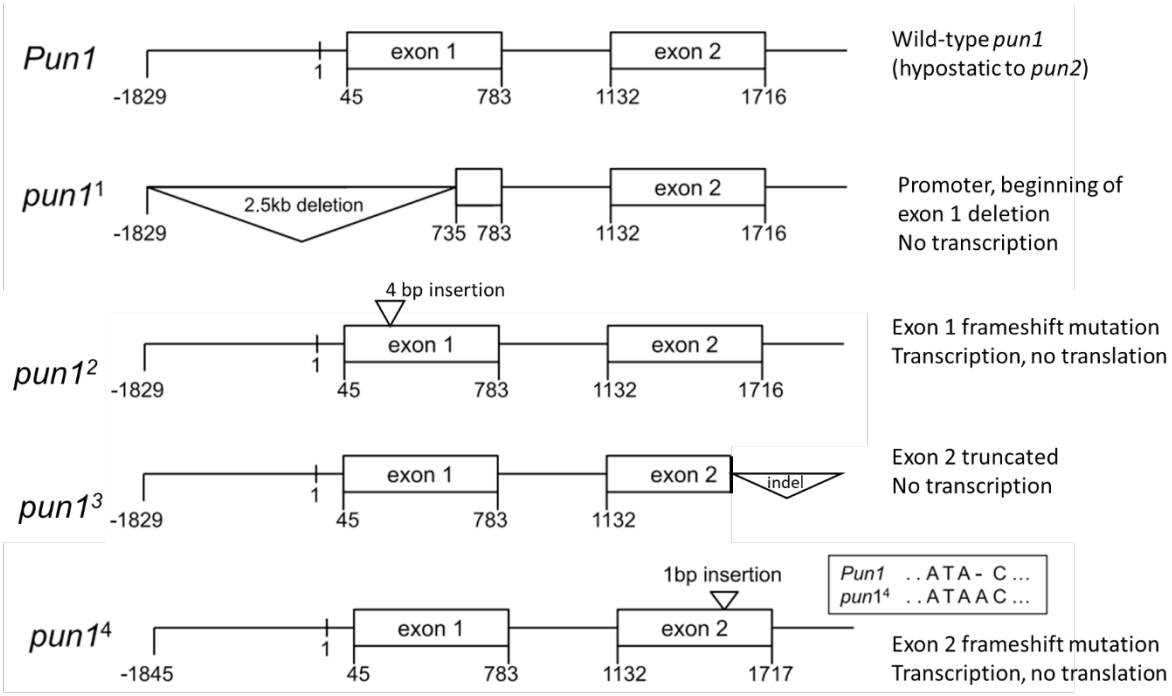

Figure 2. *Known alleles of Pun1 gene*

A large 5' deletion produced *pun1*^{1}, a frameshift mutation in the second exon is found in *pun1*^{2} and an indel truncates the second exon of *pun1*^{3}. The *pun1*^{1} and *pun1*^{3} mutations are not transcribed, while transcription but not translation has been observed for *pun1*^{2}. *pun1*^{4} possesses a single nucleotide insertion in the 2nd exon, which causes a frameshift mutation (Kirii, E. et al. 2017; Stellari et al., 2010).

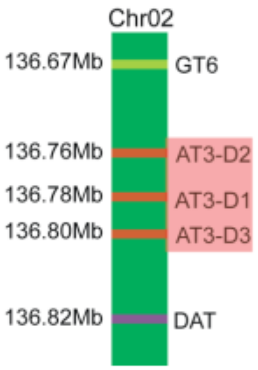

Figure 3. *Localization of the three duplicated copies of AT3 (Pun1) gene (Qin, C. et al. 2014)*

It was later found that the loss of pungency phenotype in *C. frutescens* also maps to *Pun1* locus and co-segregates with molecular markers developed to detect alleles of *Pun1*. Although this mutation is allelic to *pun1*^{1} and *pun1*^{2} more detailed studies demonstrated this to be a different allele, which was designated *pun1*^{3}. Similarly to *pun1*^{1} and *pun1*^{2} the presence of mutated allele *pun1*^{3} is correlated with loss of pungency. The allele *pun1*^{3} of *C. frutescens* has a large deletion in the 2nd exon region leading to the loss of 70 amino acids in the Pun1 protein (Stellari, G.M. et al. 2010). This allele is neither transcribed nor translated.

At *pun1*^{4} one insertion in the 2nd exon causes a frameshift mutation but it has a complete promoter and exon 1. The origin of this allele in *C. annuum* cv. 'Nara Murasaki' is unknown (Kirii, E. et al. 2017) and the variations in the loss-of-function allele have not been fully understood.

Mutations in *Pun1* locus have, for a long period of time, been preferred and utilized in the breeding of non-pungent peppers (Deshpande, R.B. 1935) due to their qualitative inheritance.

With the advancement of sequencing technology the genome of *Capsicum* sp. has also been researched (Kim, S. et al. 2014; Qin, C. et al. 2014) it appeared that three tandem copies of AT3 (*Pun1*) gene could be identified (Qin, C. et al. 2014) (Fig. 3).

Notably, only the AT3-D1 gene of the non-pungent peppers carries the *Pun1* allele (Figure 4). Authors concluded that the expression of AT3-D2 could probably keep the trace amount of capsaicin and dihydrocapsaicin detected in nonpungent peppers. The dosage compensation effect by AT3-D2 (Capang02g002091) and AT3-D1 (Capang02g002092) in locus C (Deshpande, 1935) was found to shape the pungent diversification in peppers.

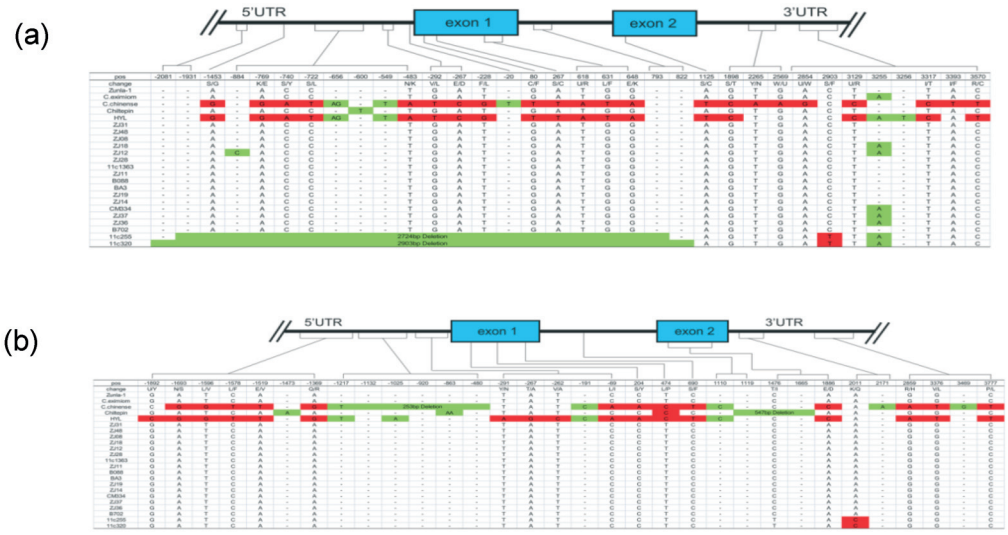

Figure 4. *Comparative analysis showed that only the AT3-D1 locus carries the pun1 mutation in non-pungent peppers (Qin, C. et al. 2014)*

(a) The structure of AT3-D1 and its haplotypes at the Pun1 locus from 20 pungent and 2 non-pungent genotypes. *pun1*^{1} allele (C locus) has a 2,724/2,930-bp deletion in non-pungent genotypes spanning the putative promoter and the first exon. (b) The structure of AT3-D2 and its haplotypes at the Pun1 locus from 20 pungent and 2 non-pungent genotypes. Note the absence of the large deletion in the non-pungent genotypes.

As early as 2007 it was suggested that the levels of expression of pungency might be modified by the presence of epistasis, over-dominance, or dominance complementation (Garcés-Claver, A. et al. 2007). Through mapping and complementation studies (Stellari, G.M. et al. 2010) the mutation causing loss of pungency in the undomesticated *Capsicum chacoense* was found at a locus PI260433-np that is not alle-

lic to the other known *Pun1* alleles. This new gene was therefore designated as *Pun2*. The further elaboration of its function by the same group demonstrated that it is epistatic to the wild type *Pun1* gene.

As the biochemical pathway of capsaicin biosynthesis is quite complex it was not surprising that (Aluru, M.R. et al. 2003) observed that transcript accumulation of several capsaicinoid biosynthetic genes was correlated with the level of pungency. A strong up-regulation of several capsaicinoid biosynthetic genes (p-AMT, Pal, Kas, BCAT, FatA – Figure 1) occurs 20 days after flowering coinciding with capsaicinoid accumulation in pungent varieties of both *C. annuum* and *C. chinense* (Stewart, C. et al. 2005). Most notably the large effects of BCAT gene (Fig. 1) that is situated at the root of the branched chain fatty acid pathway were detected (Ben-Chaim, A. et al. 2006). Another key player in the same branch was identified to be the Kas – a key to regulate the major precursors for acyl moieties of capsaicinoids (Reddy, U.K. et al. 2014).

Another important player in capsaicinoid biosynthesis is p-AMT gene. Loss-of-function mutation in p-AMT switches the capsaicinoid pathway to the capsinoid pathway, and reverses the ratio between the two analogs (Tanaka, Y. et al. 2014) thus reducing pungency.

Of course the induction and regulation of the many genes involved is expected to be controlled by various transcription factors and two of them (Erf and Jerf) were recently identified (Keyhaninejad, N. et al. 2014). The characterization of these transcription factors in nine chili cultivars with distinct capsaicinoid contents demonstrated a correlation of their expression with pungency. Interestingly, a number of amino acid variants were observed in both transcription factors from different chili cultivars, but none of these changes involved the DNA binding domains. It can therefore be expected that the observed changes in gene expression are due rather to protein-protein interactions of these transcription factors with other players, affecting capsaicinoid biosynthesis, than to direct modifications of their DNA-binding affinity.

Overall, the orchestrated induction of gene expression at about 20 DPA was found to happen downstream of Pal (Stewart, C. et al. 2007). This indicates that coordinated transcriptional regulation of capsaicinoid biosynthesis occurs with genes that are specific to this metabolic pathway.

CONCLUSSIONS

The capsaicinoids are secreted from glandular epidermal cells into subcuticular cavities that swell to form blisters along the epidermis. The blister development is positively associated with capsaicinoid accumulation and they are not present in non-pungent fruits.

Throughout more than a century of extensive research, it was established that the *pun1*^{1} allele is responsible for non-pungency within *C. annuum* as a result of a large deletion at *Pun1* that had been conserved and propagated for several centuries. It was probably the first to be identified by humans and current understanding of its function is consistent with the possibility that a single defect has pleiotropic effects on capsaicinoid biosynthesis and blisters. This implies that capsaicinoid biosynthesis is required for blister formation.

The identification of three more alleles in the same locus (*pun1*^{2}, *pun1*^{3} and *pun 1*^{4}) confirmed both the key role of *Pun1* gene and the fact that people were re-discovering new sweet varieties throughout time and space. As the interest was present not only for the fruits that completely lack pungency, but also for the ones with modified levels of it, genotypes having such properties were also developed. As the levels of capsaicinoid accumulation are quantitatively controlled, the variations in efficiency of transcription factors' binding to other protein moieties as well as the gene(s) with epistatic effects were subsequently selected long before our understanding of the genome functioning was able to reveal the exact causes of specific levels of pungency in different varieties.

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